

Taxonomic Status of the Spot-legged Treefrog in Southern Yunnan, Inferred from Mitochondrial DNA Sequences

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Abstract Populations of the spot-legged treefrogs (*Polypedates megacephalus*) in China show significant morphological variation, but no has yet been conducted to investigate the correlation between morphological variation and genetic/ecological divergence. In this study, mitochondrial DNA sequences from the 12S rRNA gene (374 bp) were amplified from 25 individual spot-legged treefrogs from southern Yunnan, China. The phylogenetic analysis using Bayesian Inference determined two haplotype clades, different from those detected by Richards and Moore (1998). Our results suggest that the phylogenetic lineages reconstructed in this study are not correlated with morphology, thus indicating that the populations in southern Yunnan may be *P. leucomystax* rather than *P. megacephalus*.

Keywords spot-legged treefrog, *Polypedates megacephalus*, *Polypedates leucomystax*, mitochondrial DNA 12S rRNA

1. Introduction

Polypedates megacephalus is a member of the poorly known *P. leucomystax* complex of cryptic species. Hallows (1861) described *P. megacephalus* as a new species based on the specimen collected from the type locality in Hong Kong, China. Matsui and Utsunomiya (1986) demonstrated that *P. megacephalus* is distinct from *P. leucomystax*. Though held as a synonym for *P. leucomystax* (named the Spot-legged Treefrog in China) by several researchers, recent records of *P. megacephalus* from northeastern India (Nagaland and Arunachal Pradesh States) might represent a different member of the *P. leucomystax* complex (Orlov *et al.*, 2001). Acoustic and karyological data for *P. leucomystax* populations from Borneo (close to the type locality of *P. leucomystax*), and Taiwan and Mainland of China indicate that the Chinese and Bornean populations were different in morphological and molecular characters, and the populations in China (Hainan and Taiwan islands), concluding that *P. leucomystax* should be *P. megacephalus* (Matsui and Utsuno-

miya, 1986). Most Chinese researchers accepted this taxonomic change (Zhao & Adler, 1993; Zhao and Yang, 1996; Fei *et al.*, 1999; Fei *et al.*, 2010; etc.), except for Yang *et al.* (1991). The phylogeographic analysis of *P. leucomystax* in Southeast Asia revealed four haplotype clades that were closely related to *P. megacephalus*, but their taxonomic relationships require further analyses (Brown *et al.*, 2010). The Global Amphibian Assessment Project (2006) recognizes both *P. megacephalus* and *P. leucomystax* in China, with their distribution boundary extending along the Red River.

In this study, the mitochondrial DNA sequences from the 12S rRNA gene were analyzed from different geographic groups to investigate the taxonomic status and phylogenetic relationship of *P. megacephalus* and *P. leucomystax*. As a marker, the mitochondrial 12S rRNA gene is commonly used to investigate interspecific phylogeny due to its low evolutionary rate (Fu, 1998).

2. Materials and Methods

A total of 25 specimens of spot-legged treefrog with different dorsum color patterns were collected from different geographic regions in China (Table 1). Two populations of *Polypedates mutus* were selected as the outgroups in the phylogenetic analysis. All animals were

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ethanized with ether and preserved in 75% alcohol. Genomic DNA was extracted from muscle or liver tissues using the standard phenol-chloroform method. The primer pair used to amplify the mitochondrial 12S rRNA gene is L1091 (5'AAA AAG CTT CAA ACT GGG ATT AGA TACCCC ACT AT 3') and H1478 (5'TGA CTG CAG AGG GTG ACG GGC GGT GTG T 3') (Kocher *et al.*, 1989):

PCR amplification was performed with a denaturation for 4 min at 95°C, followed by 39 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 50°C, and extension for 1 min at 72°C, and was terminated with a post-extension of 10 min at 72°C. PCR sequences were determined using a BigDye Terminator Kit (Perkin-Elmer) in both directions and analyzed with an ABI 377 automated sequencer (Applied Biosystems, Costa Mesa, CA) and the DNASTAR (DNASTAR Inc.). Sequence variants were rechecked by comparing the four-color electromorph of sequencing data against the computer results.

In addition, we also retrieved two mitochondrial 12S rRNA sequences of *P. megacephalus* in Taiwan, China and

P. leucomystax in the Philippines from GenBank (Richards and Moore, 1998) which were included in the analysis.

3. Results

A total 374 bp of mitochondrial DNA sequences of 12S rRNA gene were amplified from all 25 samples. Two haplotype clades were determined in the spot-legged treefrog populations in China, haplotype I from the Nangongshan population and haplotype II from all other populations. The numbers of Ti (Transitions) and Tv (Transversions) in Haplotype I and II, *P. mutus*, *P. megacephalus* and *P. leucomystax* based on sequence pairwise comparison are presented in Table 2.

The Bayesian Inference tree (unrooted) derived from the population genetics analysis using MrBayes v3.0 (Huelsenbeck and Ronquist, 2001) revealed that Haplotype I was phylogenetically different from Haplotype II. Haplotype I was closely related to *P. megacephalus* in Taiwan, China, while Haplotype II showed a close phylogenetic relationship with *P. leucomystax* in the Philippines.

Table 1 Samples used in this study

Species	Sample code	Locality	Sample size
<i>P. mutus</i>	HK1	Nanxi Township, Hekou County, Yunnan	1
	NG1-NG3	Nangongshan, Menna County, Yunnan	3
Spot-legged treefrog (“X” on back)	HK2-HK3	Nanxi Township, Hekou County, Yunnan	2
	ML1-ML3, ML8	Mengna Nature Reserve, Mengna County, Yunnan	4
	ZH1-ZH3	Xishuangbanna Tropical Botanic Garden, Chinese Academy of Sciences, Mengna County, Yunnan	3
	NG4-NG7	Nangongshan, Mengna County, Yunnan	4
Spot-legged treefrog (Strips on back)	ML4-ML7	Mengna Nature Reserve, Mengna County, Yunnan	4
	ZH4-ZH7	Xishuangbanna Tropical Botanic Garden, Chinese Academy of Sciences, Mengna County, Yunnan	4

Table 2 Numbers of Ti (Lower-left) and Tv (Up-right) of sequences based on pairwise comparisons

	Haplotype I	Haplotype II	<i>P. mutus</i>	<i>P. megacephalus</i> (Taiwan, China)	<i>P. leucomystax</i> (The Philippines)
Haplotype I		8	2	8	7
Haplotype II	16		3	15	0
<i>P. mutus</i>	13	11		10	5
<i>P. megacephalus</i> (Taiwan, China)	8	25	18		15
<i>P. leucomystax</i> (The Philippines)	16	6	19	23	

4. Discussion

The populations of *P. megacephalus* and *P. leucomystax* show different dorsum color variations, but there are no other morphological differences within the two morphotypes. For example, some populations have a black “X” on the dorsum, while some are found with several dark strips, and some with a mixed color pattern of ‘X’ and dark strips on the back. However, the phylogenetic analysis suggested that the populations of *P. leucomystax* consists of two haplotype lineages, which are phylogenetically different from *P. megacephalus*. These observations suggest that the color patterns are not correlated with the geographical distribution and could not be used to distinguish *P. megacephalus* and *P. leucomystax*.

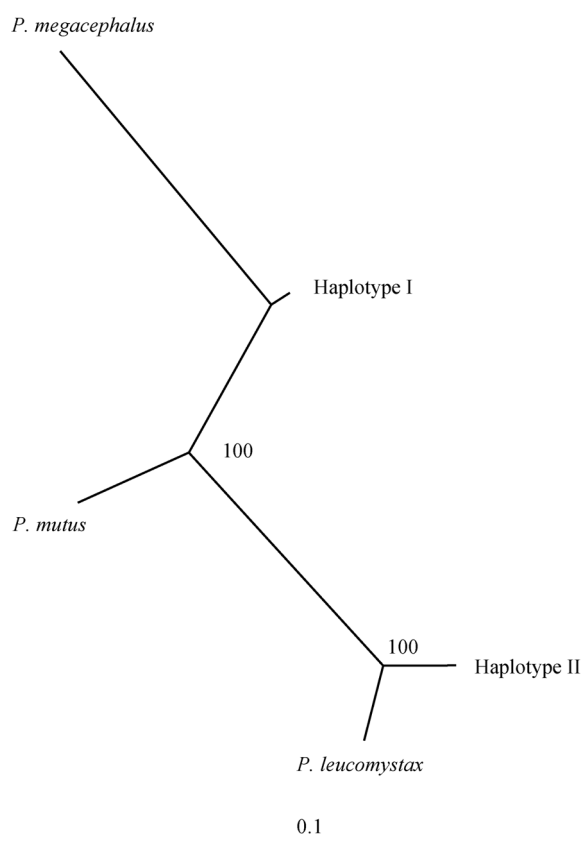


Figure 1 Phylogenetic relationships of the populations of *P. leucomystax* in China (Haplotype II and II) and the Philippines, *P. mutus* and *P. megacephalus* in Taiwan, China derived from Bayesian analysis based on 12S rRNA gene sequences. Posterior probability values are shown at the corresponding nodes. Likelihood settings from the best-fit model (TrN+G, $-\ln L=788.7644$) selected by BIC in Modeltest 3.7 (Posada and Crandall, 1998). Base=(0.3351 0.2185 0.2003), Nst=6, Rmat=(1.0000 3.9758 1.0000 1.0000 7.4288), Rates=gamma, Shape=0.2564, Pinvar=0.

Haplotype II contains most individuals of the spot-legged treefrog in southern Yunnan and is closely related with *P. leucomystax* in the Philippines (Figure 1). The small sequence variation (1.6%) suggests that the populations in Haplotype II might be *P. leucomystax*, indicating that *P. leucomystax* is distributed both east and west of the Red River.

Populations in Haplotype I are sympatrically distributed in the geographic regions of *P. leucomystax* as described through the Global Amphibian Assessment website (2006), but Haplotype I is clustered with *P. megacephalus* (Figure 1). The pairwise comparison revealed a low sequence level variation within sampling localities of Haplotype I and 4.28% sequence variation between Haplotype I and *P. megacephalus*. Therefore, the problematic identification of these lineages, either the same species, subspecies, or synonyms, will be considered further with inclusion of more molecular data.

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References

- Brown R. M., Linkem C. W., Siler C. D., Sukumaran J., Esselstyn J. A., Diesmos A. C., Iskandar D. T., Bickford D., Evans B. J., McGuire J. A., Grismer L., Supriatna J., Andayani N. 2010. Phylogeography and historical demography of *Polypedates leucomystax* in the islands of Indonesia and the Philippines: Evidence for recent human-mediated range expansion? *Mol Phylogenet Evol*, 57: 598–619
- Fei L. 1999. Atlas of Amphibians of China. Zhengzhou, China: Henan Scientific and Technologic Publishing House (In Chinese)
- Fei L., Ye C. Y., Jiang J. P. 2010. Colored Atlas of Chinese Amphibians. Chengdu, Sichuan: Sichuan Publishing House of Science & Technology (In Chinese)
- Fu J. 1998. Toward the phylogeny of the family Lacertidae: Implications from mitochondrial DNA 12S and 16S gene sequences (Reptilia: Squamata). *Mol Phylogenet Evol*, 9(1): 118–130
- Kocher T. D., Thomas W. K., Meyer A., Edwards S. V., Paabo S., Villablanca F. X., Wilson A. C. 1989. Dynamics of mitochondrial DNA evolution in animals: Amplification and sequencing with conserved primers. *Proc Natl Acad Sci U S A*, 86: 6196–6200
- Huelsenbeck J. P., Ronquist F. 2001. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics*, 17: 754–755
- Matsui M., Seto T., Utsunomiya T. 1986. Acoustic and karyotypic evidence for specific separation of *Polypedates megacephalus* from

- P. leucomystax*. *J Herpetol*, 20(4): 483–489
- Orlov N. L., Lathrop A., Murphy R. W., Cuc H. T.** 2001. Frogs of the family Rhacophoridae (Anura: Amphibia) in the northern Hoang Lien mountains (Mount Fan Si Pan, Sa Pa District, Lao Cai Province), Vietnam. *Russian J Herptol*, 17–44
- Posada D., Crandall K. A.** 1998. Modeltest: Testing the model of DNA substitution. *Bioinformatics*, 14: 817–818
- Richards C. M., Moore W. S.** 1998. A molecular phylogenetic study of the old world treefrog family Rhacophoridae. *Herpetol J*, 8: 41–46
- Yang D. T., Li S. M., Liu W. Z., Lu S. Q.** 1991. The Amphibian-Fauna of Yunnan. Beijing: China Forestry Publishing House (In Chinese)
- Zhao E. M., Yang D. T.** 1996. Amphibians and Reptiles of the Hengduan Mountains Region. Beijing: Science Press (In Chinese)
- Zhao E. M., Adler K.** 1993. Herpetology of China. Society for the Study of Amphibians and Reptiles in Cooperation with Chinese Society for the Study of Amphibians and Reptiles, Oxford, Ohio, 522 pp